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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Estrogen plays an important role in the normal breast and breast cancer development. Estrogens exert their cellular effects through ER that is a member of nuclear receptor superfamily. PRIP (Peroxisome proliferator receptor interacting protein) is a nuclear receptor coactivator that is amplified and overexpressed in breast cancer. The proposal was to investigate how the PRIP dysregulation contributes to abnormal growth and neoplastic development of breast. During the first year of the award, we have generated transgenic mice with integrated MMTV-PRIP lineages and heterozygous mice with conditional inactivated PRIP gene. About fifty estrogen-inducible genes have been identified by microarray hybridization. PRIP overexpression and amplification were found in about 60% and 10% of the breast cancers, respectively. PIMT (PRIP interacting protein with a methyltransferase domain) which interacts with and enhances nuclear receptor coactivator PRIP function was identified. PRMT2(protein arginine methyltransferase 2) was found to act as a coactivator for estrogen receptor alpha. These findings not only advanced our understanding of estrogen signaling pathway but also suggested that nuclear receptor coactivator PRIP plays an important role in the breast cancer development.

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INTRODUCTION

Estrogens plays an important role in the normal breast can breast cancer development. Estrogens exert their cellular effects through ER that is a member of nuclear receptor superfamily. Nuclear receptors including estrogen receptor achieve transcriptional activation through the participation of additional factors known as nuclear receptor coactivators. PRIP (Peroxisome proliferator receptor interacting protein) is a nuclear receptor coactivator that is amplified and overexpressed in breast cancer. The proposal was to investigate how the PRIP dysregulation contributes to abnormal growth and neoplastic development of breast. Specifically, transgenic animal models overexpressing PRIP would be developed to determine if overexpression of PRIP is sufficient to cause abnormal growth of mammary glands and if such growth ultimately leads to the development of breast tumors. PRIP transgenic mouse and PRIP null mutation mouse would be used to examine the role of coactivator PRIP in determining the susceptibility of breast cancer development induced by estrogen and chemical carcinogen. The expression of estrogen responsive genes would be examined to test the role of PRIP as an estrogen receptor coactivator in vivo and identify the estrogen responsive genes that are specifically affected by PRIP overexpression and null mutation. The prevalence of PRIP upregulation and amplification in breast cancers would be examined in 300 breast cancer samples. The potential polymorphisms in PRIP genes would be identified and the linkage between the identified polymorphisms and breast cancer risk would be elucidated by examining a large number of normal female and breast cancer patients. The proposed studies would generate novel information regarding the role of nuclear receptor coactivator in breast cancer development and the importance of the variation of nuclear receptor coactivator in the etiology of breast cancer, and most likely provide new insights regarding prevention and treatment strategies.

BODY

Task 1a. Generation of transgenic animal models overexpressing PRIP to determine if overexpression of PRIP is sufficient to cause abnormal growth of mammary glands and if such growth ultimately leads to the development of breast tumors.

We have made a construct MMTV-PRIP (Fig.1) to overexpress PRIP in mammary gland. We then used this construct for microinjection. After the plasmid was linealized, the DNA fragment was purified and microinjected into the pronucleus of F1 (C57BL/6xDBA/2) fertilized mouse embryos which were transplanted into pseudopregnant surrogate mice. Transgenic founders were identified by PCR with primers recognizing the promoter region of the transcription unit. 7 transgenic mouse lineages were identified. The founder mice were bred with C57BL/6 mice to generate F1 progeny.

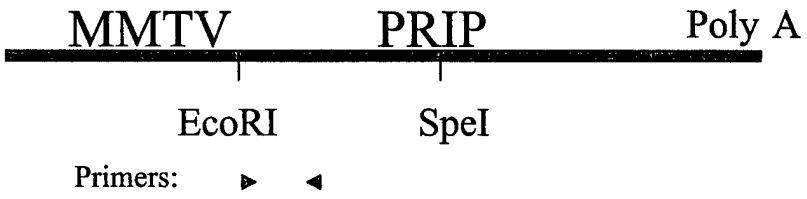

Fig. 1. MMTV-PRIP

Task 1b. Investigation of the role of coactivator PRIP in the susceptibility for breast cancer development to estrogen and chemical carcinogen using PRIP transgenic mouse and PRIP null mutation mouse.

We isolated a mouse P1 genomic clone which covers the entire PRIP gene and found that PRIP is ~ 47.5 kb in length consisting of 13 exons. The DNA from the P1 clone was used to construct the gene targeting vector loxP-PRIP(Fig. 2). Exons 8 and 9 was flanked with two loxP sites in the introns. Cre recombinase would delete these two exons leading to frame shift mutation after exon 9. The selective gene Neo was also flanked with two loxP sites. The targeting vector was then linearized and tansfected into 129/Sv derived ES cells by electroporation. ES cells were selected positively by G418 and negatively by ganciclovir. After 10 days of selection, the surviving colonies were picked up and screened by PCR. Two positive clones were isolated and further confirmed by Southern blotting. ES cells with conditional allele were injected into 3.5-day old blastocysts and implanted into a pseudopregnant foster mother to produce chimeric mice. As 129/Sv ES cells have an agouti coat color genotype, and the C57BL6 embryo is black, the contribution of 129/Sv ES cells to the chimera can be judged by the percentage of agouti coat color. Chimeric males with a high contribution of 129/Sv genetic background were mated with C57BL/6 females to obtain the germ line transmission. The agouti offspring carried one of the two alleles from 129/Sv. Heterozygotes $PRIP^{+/loxP}$ was identified by performing PCR with the tail DNA from the agouties. We have obtained three male heterozygotes $PRIP^{+/loxP}$.

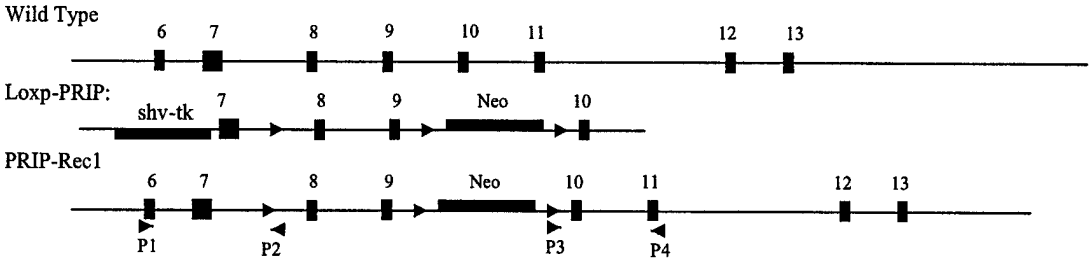

Fig.2. LoxP-PRIP constructs and targeting strategy. Schematic representation of the mouse PRIP gene, targeting vector, and the structure of the locus after gene targeting. Rec1 represents the locus after recombination. The locations of primers used for screening the recombinant ES cell are indicated.

Task 1c. Examination of the expression of estrogen responsive genes and identification of the estrogen responsive genes that are specifically affected by PRIP overexpresssion and null mutation.

Mammary glands were isolated from *ovariectomized* animals treated with 17β-estradiol or ICI 182,780 for three days. Total cellular RNA was prepared from the mammary glands and poly(A)$^+$ RNA was isolated. cDNA probe was made from poly A$^+$ RNA for hybridization to Mouse cDNA microarray filters as recommended by manufacturer (Affymatrix). The filters were then exposed to a phosphor imaging system and analyzed using software to obtain the relative expression level of each gene. We found that estrogen treatment increased the mRNA levels of about fifty genes.

We identified PIMT (PRIP interacting protein with a methyltransferase domain) which interacts with and enhances nuclear receptor coactivator PRIP function(1). We also found that PRMT2(protein arginine methyltransferase 2) to act as a coactivator for estrogen receptor α(2).

Task 2. Determination of the prevalence of PRIP upregulation and amplification in breast cancers.

We have collected about 80 breast cancer specimen from the Department of Pathology, Robert Lurie cancer center. RNA and DNA were isolated from the collected tumor samples. By using ribonuclease protection assay and Southern blot, we found about 60% of the breast cancer have PRIP overexpression and 10% have PRIP gene amplification. The overexpression of PRIP was confirmed by examining the protein by immunocytochemistry.

Task 3. Investigation of the polymorphism of PRIP gene in Breast cancer.

18 PCR were performed to cover all exons and 500 bp promoter region of PRIP gene. The PCR products were analyzed by SSCP(single strand conformational polymorphism). By examining 6 females, we found polymorphism A to G at nucleotide 289, G to A at nucleotide 2408.

KEY RESEARCH ACCOMPLISHMENTS

* Transgenic mice with integrated MMTV-PRIP lineages were generated.
* Heterozygous mice with conditional inactivated PRIP gene was created.
* About fifty estrogen-inducible genes were identified.
* PRIP overexpression and amplification were found in about 60% and 10% of the breast cancers, respectively.
* PIMT (PRIP interacting protein with a methyltransferase domain) which interacts with and enhances nuclear receptor coactivator PRIP function was identified.
* PRMT2(protein arginine methyltransferase 2) was found to act as a coactivator for estrogen receptor α.

REPORTABLE OUTCOMES

1. Zhu YJ, Qi C, Cao WQ, Yeldandi AV, Rao MS, and Reddy JK. Cloning and characterization of PIMT, a protein with a methyltransferase domain, which interacts with and enhances nuclear receptor coactivator PRIP function. *Proc. Natl. Acad. Sci. USA* 98: 10380-10385, 2001.
2. Qi C, Chang J, Zhu YW, Yeldandi AV, Rao MS and Zhu YJ. Identification of PRMT2 as a coactivator for estrogen receptor alpha. *J. Biol. Chem. (in press).* 2002

CONCLUSIONS

We have created MMTV-PRIP transgenic mice and heterozygous mice with conditional inactivated PRIP gene which will finally help to reveal the function of this important coactivator. We isolated PIMT and PRMT2 as two components in the estrogen receptor transcriptional activation, advancing our understanding of estrogen signaling pathway. The finding that PRIP overexpression occurs in about 60% and gene amplification occurs 10% of the breast cancers suggested that this coactivator plays an important role in the breast cancer development.

REFERENCES

1. Zhu YJ, Qi C, Cao WQ, Yeldandi AV, Rao MS, and Reddy JK. Cloning and characterization of PIMT, a protein with a methyltransferase domain, which interacts with and enhances nuclear receptor coactivator PRIP function. *Proc. Natl. Acad. Sci. USA* 98: 10380-10385, 2001.
2. Qi C, Chang J, Zhu YW, Yeldandi AV, Rao MS and Zhu YJ. Identification of PRMT2 as a coactivator for estrogen receptor alpha. *J. Biol. Chem. (in press).* 2002.

APPENDICES

Two articles are attached.

Cloning and characterization of PIMT, a protein with a methyltransferase domain, which interacts with and enhances nuclear receptor coactivator PRIP function

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The nuclear receptor coactivators participate in the transcriptional activation of specific genes by nuclear receptors. In this study, we report the isolation of a nuclear receptor coactivator-interacting protein from a human liver cDNA library by using the coactivator peroxisome proliferator-activated receptor-interacting protein (PRIP) (ASC2/AIB3/RAP250/NRC/TRBP) as bait in a yeast two-hybrid screen. Human PRIP-interacting protein cDNA has an ORF of 2,556 nucleotides, encodes a protein with 852 amino acids, and contains a 9-aa VVDAFCGVG methyltransferase motif I and an invariant GXXGXXI segment found in K-homology motifs of many RNA-binding proteins. The gene encoding this protein, designated PRIP-interacting protein with methyltransferase domain (PIMT), is localized on chromosome 8q11 and spans more than 40 kb. PIMT mRNA is ubiquitously expressed, with a high level of expression in heart, skeletal muscle, kidney, liver, and placenta. Using the immunofluorescence localization method, we found that PIMT and PRIP proteins appear colocalized in the nucleus. PIMT strongly interacts with PRIP under *in vitro* and *in vivo* conditions, and the PIMT-binding site on PRIP is in the region encompassing amino acids 773–927. PIMT binds *S*-adenosyl-L-methionine, the methyl donor for methyltransfer reaction, and it also binds RNA, suggesting that it is a putative RNA methyltransferase. PIMT enhances the transcriptional activity of peroxisome proliferator-activated receptor γ and retinoid-X-receptor α, which is further stimulated by coexpression of PRIP, implying that PIMT is a component of nuclear receptor signal transduction apparatus acting through PRIP. Definitive identification of the specific substrate of PIMT and the role of this RNA-binding protein in transcriptional regulation remain to be determined.

The nuclear receptor superfamily consists of members of ligand-regulated transcriptional factors comprising receptors for steroid and thyroid hormones, vitamin D_3, retinoic acid, and peroxisome proliferators, among others (1, 2). All nuclear receptors share a common structure with a highly conserved DNA-binding domain consisting of two zinc fingers, a C-terminal hormone-binding domain, and two transcriptional activation function (AF) domains, termed AF-1 in the N-terminal domain and AF-2 in the hormone-binding domain (3). Transactivation of the AF-2 domain is ligand-dependent and can be blocked by the binding of antagonists. The activities of AF-1 and -2 vary depending on responsive cell types as well as a short DNA sequence, termed response element, located in the promoter regions of target genes (2, 3).

The molecular mechanism(s) by which nuclear receptors achieve transcriptional activation in a tissue-specific fashion is not fully understood. The current models call for the participation of additional factors for modifying the chromatin structure and mediating the interaction between the activated nuclear receptor and the basal transcriptional machinery in a ligand-dependent fashion. During the past few years, many nuclear receptor-binding proteins have been identified (4, 5). Most of them contain the conserved LXXLL (where L is leucine and *X* is any amino acid) signature motif, which mediates recognition of nuclear receptors (4). The nuclear receptor coactivators identified to date include the well-characterized steroid receptor coactivator-1 (SRC-1) family with three members [SRC-1 (6), TIF-2 (SRC-2, GRIP1) (7), p/CIP (ACTR, AIB1, RAC3, and SRC-3) (8–10)], CREB-binding protein (CBP)/p300 (11), PPAR-binding protein (PBP) (12), and PPARγ coactivator-1 (PGC-1) (13). SRC-1 and CBP/p300 possess intrinsic histone acetyltransferase activity and also recruit other proteins with histone acetyltransferase activity, indicating their role in chromatin modification (2, 4, 8, 14). In addition to histone acetylation, there is emerging evidence that suggests a role for histone methylation in the regulation of nuclear receptor transcriptional activity. A protein, designated as coactivator-associated arginine methyltransferase 1 (CARM1), which associates with SRC-2, can methylate histones, and the methyltransferase activity of CARM1 appears necessary for this protein to potentiate nuclear receptor activity (15). Furthermore, protein arginine methyltransferase 1, which shares a region of homology with CARM1, appears to enhance transcriptional activity by acting synergistically with CARM1 (16). Recent evidence suggests that coactivator proteins form distinct coactivator complexes, such as the one anchored by CBP/p300 that integrates the SRC-1 family of proteins and p/CAF to carry out histone acetyltransferase reactions, and the other thyroid hormone receptor-associated protein (TRAP)/vitamin D receptor-interacting protein (DRIP)/activator-recruited cofactor (ARC) complex anchored by PBP (TRAP220/DRIP205) (12, 17–19), which links to the basal transcription machinery (4, 20). PBP, with no histone acetyltransferase activity, serves as an anchor protein to recruit the multiprotein complex that appears to act more directly on the transcriptional apparatus, presumably after the unwinding of chromatin facilitated by the CBP/p300 anchored multiprotein complex (4). In agreement with the central role of CBP/p300 and PBP in the coactivator complex configuration and in nuclear receptor-mediated transcriptional activity, knockout experiments revealed that both CBP, p300, and PBP null mutations

Abbreviations: PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; SRC-1, steroid receptor coactivator-1; CARM1, coactivator-associated arginine methyltransferase 1; PRIP, PPAR-interacting protein; PIMT, PRIP-interacting protein with methyltransferase domain; EST, expressed sequence tag; RXR, retinoid-X-receptor for 9-*cis*-retinoic acid; GST, glutathione *S*-transferase; AdoMet, *S*-adenosyl-L-methionine; PBP, PPAR-binding protein.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY028423 and AF389908).

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lead to embryonic lethality (21–26), indicating that disruption of these pivotal anchoring coactivators affects the function of many nuclear receptors and possibly other transcription factors.

In this work, we report the cloning of a protein that binds the recently identified nuclear receptor coactivator peroxisome proliferator-activated receptor-(PPAR) interacting protein (PRIP) (ASC2/AIB3/RAP250/NRC/TRBP) (27–31). We cloned PRIP by using PPARγ as bait in the yeast two-hybrid system (27); others have cloned this coactivator by using thyroid hormone receptor and retinoid-X-receptor for 9-*cis*-retinoic acid (RXR) (28–31). To elucidate the role of PRIP, we set out to isolate the PRIP-interacting protein(s) with a two-hybrid system. One of the proteins identified is designated PRIP-interacting protein with methyltransferase domain (PIMT); this protein is a ubiquitously expressed nuclear protein containing a methyltransferase domain. PIMT is able to enhance the transcriptional activity of PPARγ and RXRα, which is further potentiated by overexpression of PRIP. We also show that PIMT binds *S*-adenosyl-L-methionine (AdoMet) and RNA, implying that PIMT may function as a putative RNA methyltransferase.

Materials and Methods

Plasmids. GAL4-PRIP (amino acids 773-2068), GAL4-PPARγ, PCMV-PRIP, PCMV-PPARγ, peroxisome proliferator response element (PPRE)-luciferase (LUC), PCMX-RXRα, and RXRE-LUC have been described elsewhere (12, 27, 32). PCMV-PIMT was constructed by inserting the full-length coding region of PIMT cDNA into *Hin*dIII/*Xho*I site of PCDNA 3.1(+) (Invitrogen). PCMV-PIMT-FLAG was made by inserting full-length PIMT cDNA into *Hin*dIII/*Bam*HI site of p3XFLAG-CMV-14 (Sigma).

Isolation of Human and Mouse PIMT cDNA. A partial cDNA encoding a protein that interacts with PRIP was first isolated by yeast two-hybrid screening of a human liver cDNA library, as described previously (12, 27). We then obtained the full-length cDNA by checking human expressed sequence tag (EST) in GenBank. The full-length cDNA we cloned has been designated PIMT to reflect its ability to bind PRIP and its putative functional domain. We then searched the mouse EST database in GenBank, by using the human PIMT cDNA sequence. Several ESTs, including ESTs covering the 5′ and 3′ of PIMT cDNA, were found. The primers 5′-GAGCCCGGATAACGAAATGT-3′ and 5′-CTTCCTCCTGTCTCTCACTT-3′ were synthesized on the basis of the EST sequences. The full-length mouse PIMT cDNA was amplified by using spleen mouse cDNA and sequenced.

Northern Blot Analysis and *in Situ* Hybridization. Human multiple tissue Northern blot (CLONTECH) containing 2 μg of poly(A) RNA in each lane was probed with ^{32}P-labeled PIMT full-length cDNA, according to the conditions outlined by the manufacturer. For *in situ* hybridization, tissues were fixed in 4% paraformaldehyde at 4°C for 14–18 h, dehydrated, embedded in paraffin, and 7-μm-thick sections were cut under ribonuclease-free conditions. RNA riboprobes (antisense and sense riboprobes) for PIMT were synthesized in the presence of digoxigenin-labeled UTP (Roche Diagnostics). Prehybridization, hybridization, washing, and immunological detection were performed as described (33).

Glutathione *S*-Transferase (GST) Pull-Down Assays. The GST alone or GST-fusion protein, GST-PRIP (amino acids 772–1317), was expressed in *Escherichia coli* BL21 and bound to glutathione-Sepharose-4B beads according to the manufacturer's instructions (Amersham Pharmacia). *In vitro* translation was performed by using rabbit reticulocyte lysate (Promega) and labeled with [^{35}S]methionine. In a GST pull-down assay, 15 μl of GST fusion protein on glutathione-Sepharose beads was incubated with 5 μl of [^{35}S]methionine-labeled *in vitro*-translated proteins for 2 h in 600 μl of 20 mM Tris·HCl, pH 7.5/100 mM KCl/0.7 mM EDTA/0.05% Nonidet P-40/1 mM phenylmethylsulfonyl fluoride (NETN). Bound proteins were washed four times with 800 μl of NETN, eluted by boiling for 2 min in 24 μl of SDS loading buffer, separated by SDS/PAGE, and autoradiographed.

Immunoprecipitation. COS-1 cells are transfected with PCMV-PIMT-FLAG by the calcium precipitation method. Twenty-four hours after transfection, the cell was harvested. The lysate was immunoprecipitated with anti-PRIP or preimmune serum. The precipitates were resolved on SDS/PAGE and subjected to Western blot analysis by using anti-FLAG (Sigma).

Cell Culture and Transfection. CV-1 cells (1×10^5) were plated in six-well plates and cultured in DMEM containing 10% FCS for 24 h before transfection. Cells were transfected for 5 h with 1.5 μg of luciferase reporter plasmid DNA, 1.5 μg of appropriate expression plasmid DNA, and 0.4 μg of β-galactosidase expression vector pCMVβ (CLONTECH) DNA by using the Lipofectamine 2000-mediated transfection method (GIBCO/BRL). Cell extracts were prepared 24 h after transfection and assayed for luciferase and β-galactosidase activities (Tropix, Bedford, MA).

Immunofluorescence. COS-1 cells were transfected with PCMV-PIMT-FLAG by using Lipofectamine 2000 (GIBCO/BRL), and 24 h after transfection, the cells were fixed in 1% formaldehyde and washed once with PBS, pH 7.4, after which autofluorescence was quenched with 50 mM ammonium chloride in PBS. Cells were washed with PBS, permeabilized with 0.5% saponin, and then blocked with 0.2% fish skin gelatin. The cells were incubated with the primary antibody anti-FLAG (Sigma) and anti-PRIP, followed by incubation with secondary antibodies. Fluorescence microscopy and digital image collection were performed by using an Olympus (New Hyde Park, NY) microscope and a photometrix cooled charge-coupled device camera driven by DELTAVISION software from Applied Precision (Seattle).

AdoMet-Binding Assay. Purified GST or GST-PIMT (10 μg) was incubated with 20 μCi of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (Amersham Pharmacia) in a buffer containing 20 mM Tris·HCl, pH 7.5, 150 mM NaCl, and 2 mM $MgCl_2$, at 37°C for 10 min. The protein was trapped on a HAWP 02500 filter (Millipore). Unbound *S*-adenosyl-L-[*methyl*-^{3}H]methionine was removed by washing with the buffer (20 mM Tris·HCl, pH 7.5/150 mM NaCl/2 mM $MgCl_2$). The filters were dried, and the amount of bound *S*-adenosyl-L-[*methyl*-^{3}H]methionine was quantified by liquid scintillation counting.

RNA-Binding Gel Retardation Assay. The degenerate ^{32}P-labeled RNA (GGAGACCGGCCAGGAUCCAAGCUNNNNNNNNCAUAAGGAUCCAAGCUAA) was generated by *in vitro* transcription (Ambion, Austin, TX). Reactions (20 μl) containing 0.1 μg of protein indicated, 1 μg of anti-FLAG, 10,000 cpm of RNA, and 1× buffer (20 mM Tris·HCl, pH 7.5/150 mM NaCl/2 mM $MgCl_2$) was assembled and incubated for 15 min on ice. The reactions were loaded on a native polyacrylamide gel (Invitrogen) running at 300 V at 4°C in 0.5× TBE buffer (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3). The gels were dried and autoradiographed.

Results

Isolation of PIMT. Using PRIP (amino acids 773-2068) as bait in a yeast two-hybrid system, we isolated from human liver cDNA library a partial cDNA encoding a PRIP-interacting protein, which was designated as PIMT. The rest of the cDNA was obtained from a human EST clone that was identified by

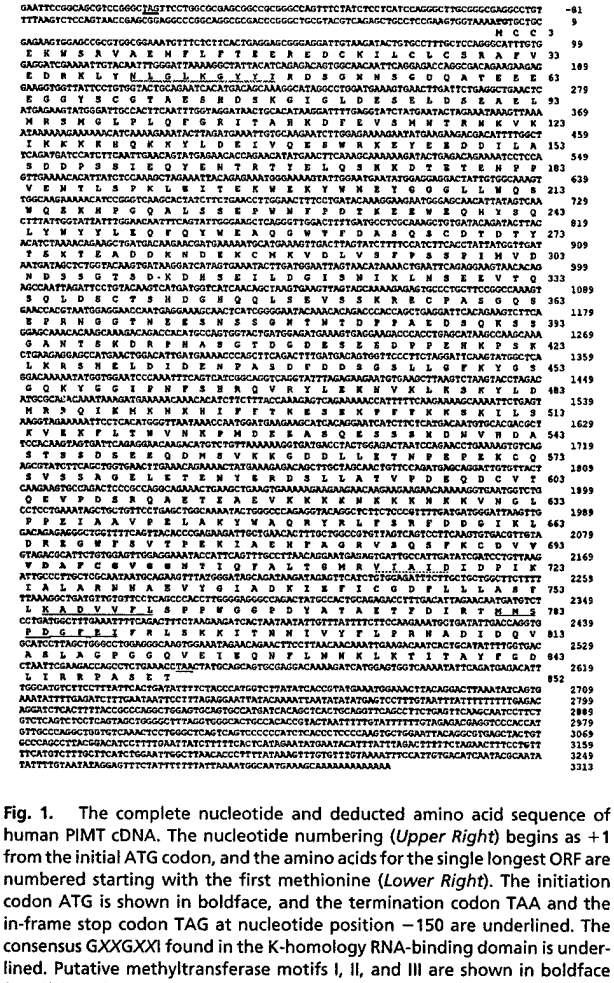

Fig. 1. The complete nucleotide and deducted amino acid sequence of human PIMT cDNA. The nucleotide numbering (*Upper Right*) begins as +1 from the initial ATG codon, and the amino acids for the single longest ORF are numbered starting with the first methionine (*Lower Right*). The initiation codon ATG is shown in boldface, and the termination codon TAA and the in-frame stop codon TAG at nucleotide position −150 are underlined. The consensus G*XXGXX*I found in the K-homology RNA-binding domain is underlined. Putative methyltransferase motifs I, II, and III are shown in boldface (motif I), underlined (motif II), and double underlined (motif III). Motif I and Post I (dotted) are known to interact directly with *S*-adenosyl-L-methionine.

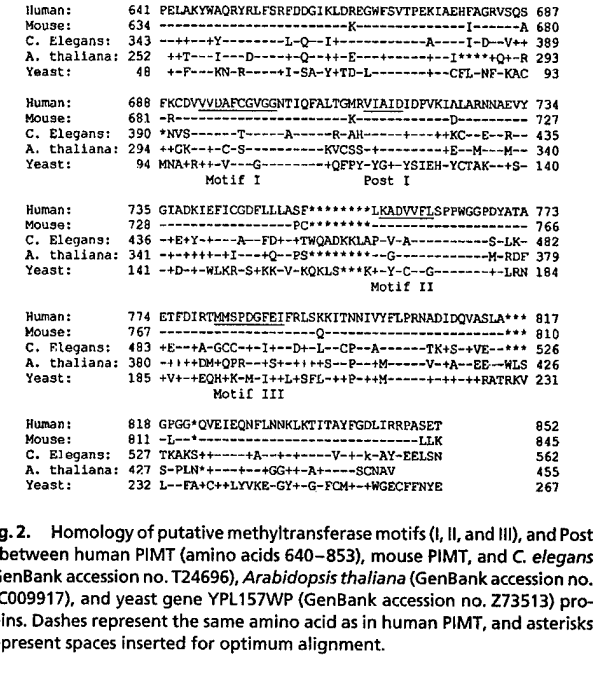

Fig. 2. Homology of putative methyltransferase motifs (I, II, and III), and Post I, between human PIMT (amino acids 640–853), mouse PIMT, and *C. elegans* (GenBank accession no. T24696), *Arabidopsis thaliana* (GenBank accession no. AC009917), and yeast gene YPL157WP (GenBank accession no. Z73513) proteins. Dashes represent the same amino acid as in human PIMT, and asterisks represent spaces inserted for optimum alignment.

searching for the homologous sequence in GenBank's human EST. The full-length human PIMT has an ORF of 2,556 nucleotides that encodes a protein of 852 amino acids (Fig. 1) with an estimated molecular mass of 96.5 kDa. The PIMT cDNA fragment directly recovered by yeast two-hybrid screening encodes amino acids from 1 to 384. The human PIMT cDNA contains a short 5′ (165-bp) and a long 3′ (743-bp) untranslated region (Fig. 1). The start of the coding sequence was defined by the first ATG downstream of an in-frame stop codon at position −150. The gene encoding human PIMT is localized on chromosome 8q11, spans more than 40 kb, and consists of more than 13 exons (data not presented). The full-length mouse PIMT cDNA we cloned is 2,648 bp in length, with an ORF of 2,559, which encodes a peptide of 853 amino acids that is 73% identical and 84% similar to the human PIMT (data not shown).

When the nucleotide-derived amino acid sequences of human and mouse PIMTs were used to search the databases, no protein that showed significant overall similarity was detected, but the carboxyl region of human PIMT from amino acids 640–852 shows 56% identity and 68% similarity of amino acids with a protein of unknown function in *Caenorhabditis elegans* and 35% identity and 52% similarity of amino acids with the YPL157WP of the yeast (Fig. 2). They most likely represent homologues of human PIMT. Protein with such a high homology is not found in *Drosophila*. In this region, the human PIMT contains VVDAFCGVG (amino acids 693–701), which is similar to the highly conserved motif I with a consensus hh(D/S)(L/P)F*X*G*X*G, where "h" represents a hydrophobic residue, and "*X*" represents any amino acid, found in a number of m^5C and m^5A methyltransferases and other enzymes that use AdoMet as substrate (34–38). The C-terminal part of human PIMT also reveals regions that are similar to methyltransferase motif II, motif III, and Post 1 (Fig. 2).

Tissue Distribution of the PIMT Transcript. Human PIMT is expressed as a 3.2-kb transcript in all tissues examined, with high levels of expression of PIMT mRNA found in heart, skeletal muscle, and kidney (Fig. 3). By *in situ* hybridization, using mouse PIMT as a probe, we found the presence of PIMT transcripts in several tissues of adult mouse, including liver parenchymal cells, proximal tubular epithelium of kidney, intestinal epithelium, exocrine and endocrine pancreatic tissues, thyroid follicles, and prostate epithelium, among others (not illustrated). To localize PIMT protein, a plasmid containing three FLAG-epitopes linked to the C-terminal portion of the PIMT protein was transfected into COS-1 cells derived from African green monkey kidney (American Type Culture Collection CRL1651). Immuno-

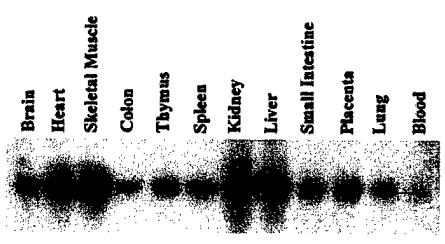

Fig. 3. PIMT mRNA expression in various human tissues. A human multiple tissue Northern blot (CLONTECH) containing 2 μg of poly(A) RNA for each tissue was probed with ^{32}P-labeled full-length human PIMT cDNA and then exposed to film at −80°C with an intensifier screen for 24 h. The transcript size of PIMT is 3.2 kb in all tissues examined.

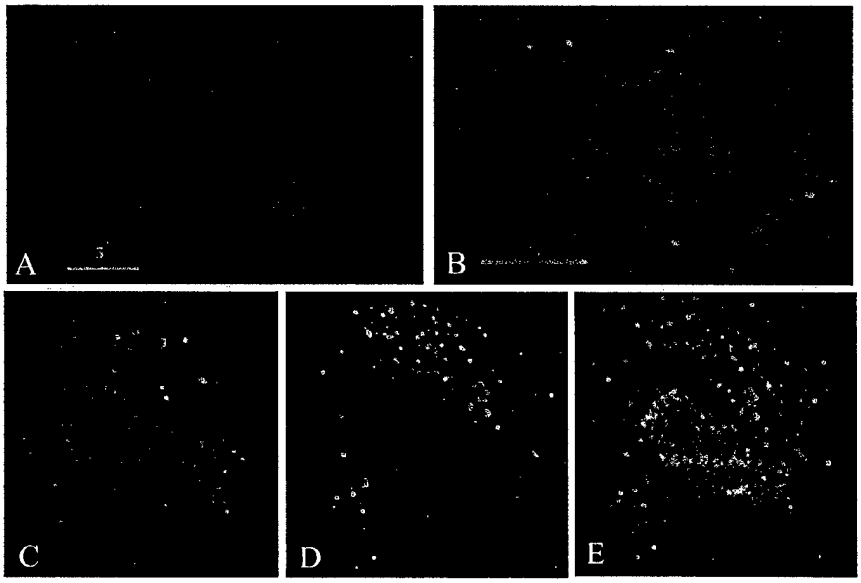

Fig. 4. PIMT and PRIP colocalize in the nucleus. PRIP was visualized by using conventional fluorescence microscopy (*A*) and by DELTAVISION deconvolution microscopy (*B*). By DELTAVISION microscopy, a speckled pattern of distribution of this coactivator is noted. PIMT was expressed transiently in COS-1 cells by using three FLAG epitopes linked to the C terminus of PIMT, and the FLAG epitope of PIMT was visualized by deconvolution microscopy by using anti-FLAG antibodies (*C*). Merging of the PIMT (*D*) and PRIP (*D*) localization images reveals overlapping localization (yellow) of these two proteins (*E*).

fluoresence with anti-FLAG revealed the expressed PIMT protein is localized in the nucleus (Fig. 4). Localization of PRIP by using anti-PRIP revealed that PRIP is also localized in the nucleus and, when the images of PIMT and PRIP localization were merged, an overlapping localization of PRIP and PIMT has been noted, suggesting colocalization of these molecules in the nucleus (Fig. 4).

Interaction of PIMT with PRIP *in Vitro* and *in Vivo*. The direct interaction between PRIP and PIMT was further tested by using *in vitro* a GST-binding assay with a bacterially generated GST-PRIP fusion protein (amino acids 773-1317) and radioactively labeled *in vitro*-translated PIMT. As shown in Fig. 5, PIMT specifically interacted with the immobilized GST-PRIP but not with GST. Using further truncated PRIP, we narrowed the PIMT-binding site on PRIP to a region encompassing amino acids 773–927 (Fig. 5*A*). To determine whether PIMT and PRIP interact within the context of intact cells, a vector encoding human PIMT with C-terminal FLAG epitopes was transfected into COS-1 cells. The potential complex between PRIP and PIMT was immunoprecipitated by using anti-PRIP, and the product was analyzed by immunoblotting by using anti-FLAG to demonstrate PIMT in the precipitate (Fig. 5*B*). The results showed that anti-PRIP precipitated PIMT but not control serum, demonstrating the existence of a PRIP and PIMT complex *in vivo* (Fig. 5*B*). Because PRIP is able to form homodimers, we examined whether PIMT can also form homodimers. A GST pull-down assay was performed with GST-PIMT fusion protein (amino acids 326–852) and *in vitro*-translated [^{35}S]methionine-labeled human PIMT. The results demonstrated that *in vitro*-translated PIMT interacts with GST-PIMT, suggesting that PIMT may form homooligomers (Fig. 5*C*).

PIMT Binds AdoMet. Human PIMT contains discernible methyltransferase motifs I, II, and III, and also a conserved motif called Post 1 (Figs. 1 and 2). Because motif I (FXGXG) is known to interact with an adenosyl moiety of the cofactor (34, 35), we assessed the ability of human PIMT to bind AdoMet by a filter-binding assay. The partial PIMT (amino acid residues 326–852), which contains the putative methyltransferase domain, binds AdoMet, whereas the N-terminal PIMT fragment (amino acids 1–384), which does not contain these motifs, failed to bind AdoMet (Fig. 6*A*).

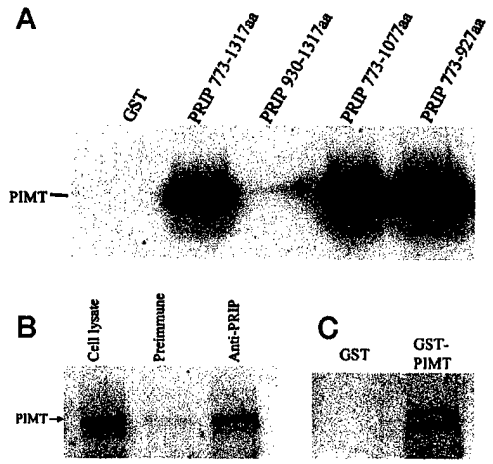

Fig. 5. *In vitro* interaction of PRIP with PIMT. (*A*) [^{35}S]Methionine-labeled full-length PIMT generated by *in vitro* translation was incubated with glutathione-Sepharose beads bound with purified *E. coli*-expressed GST-PRIP (amino acids 773-1317, 930-1317, 773-1077, and 773–927) or GST. The bound proteins were eluted and analyzed by using 10% SDS/PAGE and autoradiographed. (*B*) Coimmunoprecipitation of PIMT and PRIP in intact cells. pcDNA3.1-FLAG-PIMT were transfected into COS-1 cells and, 24 h after transfection, cells were harvested. Cell lysate was immunoprecipitated with anti-PRIP or control serum, and immunoprecipitates were subjected to immunoblotting with anti-FLAG. Epitope-tagged protein PIMT can be coprecipitated by anti-PRIP (lane 3) but not by preimmune serum (lane 2). Lane 1, 1/20 input cell lysate. (*C*) PIMT forms homodimers. GST-PIMT (326-852 aa) or GST alone was incubated with PIMT labeled with [^{35}S]methionine. The bound protein was electrophoresed and visualized by fluorography. GST-PIMT, but not GST alone, binds PIMT.

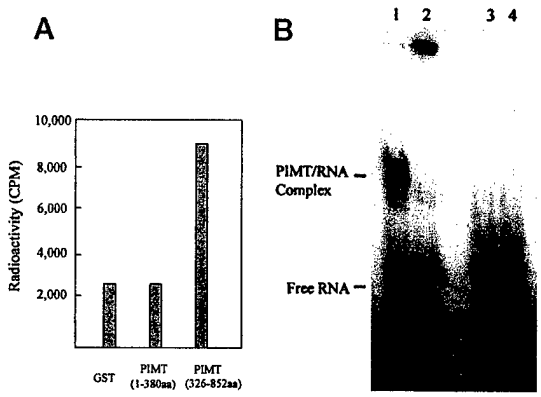

Fig. 6. PIMT binds AdoMet and RNA. (*A*) PIMT binds AdoMet. Purified protein was incubated with *S*-adenosyl-L-[*methyl*-^{3}H]methionine, and the amount of protein bound to AdoMet was trapped on a filter and quantified by liquid scintillation. GST-PIMT (amino acids 326–852), which contained the methyltransferase domain, retained a significant amount of labeled AdoMet, whereas the filter with GST-PIMT (amino acids 1–384) showed background radioactivity as that with GST alone. (*B*) PIMT is an RNA-binding protein. ^{32}P-labeled RNA produced by *in vitro* transcription is incubated with purified FLAG-PIMT (amino acids 1–384). A PIMT–RNA complex is seen in addition to the free RNA (lane 1). The addition of anti-FLAG markedly diminished the complex; instead, antibody and PIMT–RNA formed a big complex, which hardly migrated into the gel (lane 2). Lanes 3 and 4 represent controls by using purified FLAG-PRIP (amino acids 786-1132) and purified FLAG-PRIP (amino acids 786-1132) plus anti-FLAG, respectively.

PIMT Is an RNA-Binding Protein. In an effort to search for the potential substrate of PIMT, we examined whether PIMT can bind to RNA by using a gel-shift assay. When purified FLAG-PIMT (amino acids 1–384) was incubated with ^{32}P-labeled RNA produced by *in vitro* transcription, a PIMT–RNA complex was produced (Fig. 6*B*, lane 1). The addition of anti-FLAG markedly diminished the formation of the PIMT–RNA complex; instead, antibody and PIMT–RNA formed a high molecular-weight complex, which failed to migrate into the gel (Fig. 6*B*, lane 2). As a control, when purified FLAG-PRIP (amino acids 786-1132) was used in an RNA-binding assay with or without anti-FLAG, no detectable RNA binding was observed (Fig. 6*B*, lanes 3 and 4). Close examination of PIMT amino acids 1–384 revealed that they contained a consensus sequence G*XX*G*XX*I (Fig. 1), which is present in virtually all K-homology domains of RNA-binding proteins (39, 40). A gel retardation experiment revealed that FLAG-PIMT (amino acids 1–384) was unable to bind single- and double-stranded DNA (data not shown).

PIMT Potentiates the Transcriptional Activity of PPARγ. To determine the functional relevance of the interaction of PIMT with PRIP, we transiently overexpressed PIMT and PRIP in CV-1 cells along with PPARγ and monitored the transcriptional activity of PPARγ with expression of the PPRE-linked reporter luciferase gene. Both PIMT and PRIP, when transfected individually, consistently increased the transcription of the PPARγ-mediated luciferase gene by about 1.6-fold in the presence of PPARγ ligand BRL49653 (Fig. 7*A*). Cotransfection of PIMT and PRIP resulted in further enhancement of ligand-dependent reporter gene expression, indicating that PIMT synergizes PRIP action. The truncated PIMT (amino acids 1–384), without methyltransferase domains, can also enhance PPARγ transcriptional activity by about 1.8-fold, suggesting the methyltransferase domain is not necessary for PIMT's ability to enhance the transcriptional activity of PPARγ under the conditions of transient transfection. Similar results were observed with RXRα (Fig. 7*B*).

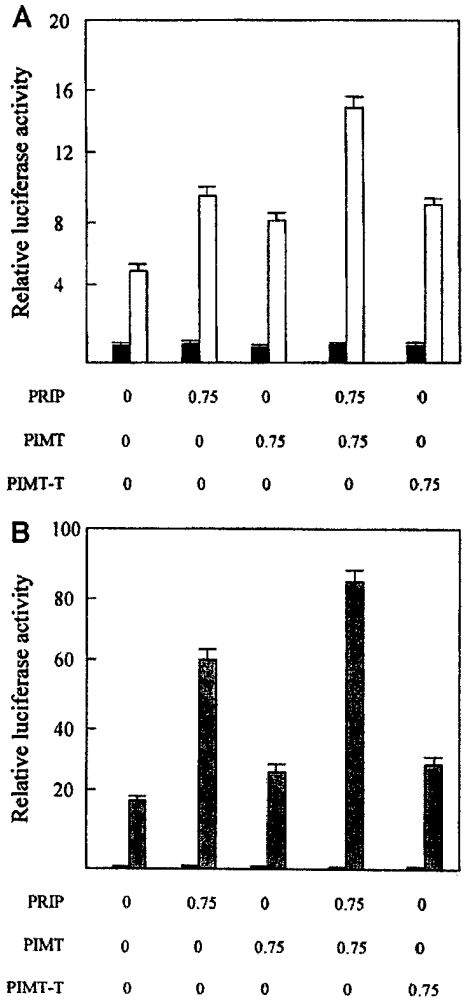

Fig. 7. PIMT increases PPARγ- and RXRα-mediated transactivation of reporter expression in CV-1 cells. (*A*) CV-1 cells were cotransfected with 1.5 μg of reporter construct PPRE-TK-LUC, 25 ng of PCMV-mPPARγ, and 0.4 μg of PCMVβ, along with the indicated plasmid in the absence (solid bar) or presence (open bar) of 10^{-5} M BRL49653. Transfection, without the indicated plasmid, was compensated by adding the same amount of PCDNA3.1. The activity obtained on transfection of PPRE-TK-LUC, without exogenous PIMT in the absence of ligand was taken as 1. Results are the mean of three independent transfections. (*B*) Transfection analysis for RXRα was performed in the same way as PPARγ, except for using RXRE-TK-LUC and PCMX-RXRα in the absence (solid bar) and presence (hatched bar) of ligand 9-*cis*-retinoic acid.

Discussion

In a previous study, using the yeast two-hybrid system, we isolated and characterized mouse PRIP as a PPAR coactivator (27). The cloning of this nuclear receptor coactivator from human and rat has also been reported (28–31). PRIP has been shown to interact with a variety of nuclear receptors and also CBP and is widely expressed with the highest expression in heart, ovary, testis, prostate, and skeletal muscle (27–31). These observations suggested this coactivator may prove as indispensable as CBP, p300, and PBP in mediating the transcriptional activity of nuclear receptors and other transcription factors. In this study, we used a two-hybrid system with PRIP as bait and isolated PIMT, a PRIP-interacting protein, which has a methyltransferase domain. The mRNA of PIMT is ubiquitously expressed, and its protein appears colocalized in the nucleus

with PRIP. It appears that both PRIP and PIMT are expressed abundantly in the same tissues and cell types, suggesting these two proteins play a synergistic function *in vivo*. The PIMT-binding region in PRIP (amino acids 773–927) has also been determined by using truncated portions of PRIP in GST pull-down assays; this region avidly binds PIMT. Overexpression of PIMT enhances the transcriptional activity of PPARγ and RXR, and this enhancement is further stimulated by overexpression of PRIP, suggesting that PIMT is a component of nuclear receptor signal transduction that acts through PRIP.

PIMT reveals the presence of an AdoMet-binding site VVDAFCGVG, which is similar to the highly conserved methylltransferase motif I with a consensus hh(D/S)(L/P)F*X*G*X*G, (where "h" represents a hydrophobic residue, and "*X*" represents any amino acid) (34, 35). This motif is found in several methyltransferases and other enzymes that use AdoMet as substrate (34–36). Of considerable interest is that the motif FCGVG present in PIMT corresponds to the sequence (F*X*G*X*G) found in many RNA and DNA methyltransferases (34, 35, 41). The Phe in this motif is known to interact with the adenosyl moiety of the cofactor AdoMet (35). In other methyltransferases, particularly in protein arginine methyltransferases, Phe is often substituted by Gly (34, 35). The binding of AdoMet, the donor of methyl groups, to purified PIMT under *in vitro* conditions essentially confirms that PIMT is a putative methyltransferase, but the target of PIMT remains unknown. Although PIMT failed to methylate *in vitro* either histone, unlike CARM1 (15), or DNA (Y.Z., unpublished work), we found that it binds RNA. The segment of PIMT (amino acids 1–384) that binds RNA contains an invariant G*XX*G*XX*I motif present in the K-homology domains of RNA-binding proteins (39, 40). It is estimated that the human genome contains ≈1,500 RNA-binding proteins, but their distribution across global, group-specific, and type-specific classes is unknown (42). PIMT may function as a putative RNA methyltransferase, raising the possibility that some coactivators and/or their cofactors may influence transcription by binding and methylating RNA. PIMT shows high homology with an uncharacterized protein YPL157W (GenBank accession no. Z73513) from yeast and a functionally unknown protein (GenBank accession no. T24696) from *C. elegans*. They most probably represent the homologues of PIMT and should have similar substrates. The yeast strain with null mutation of YPL157W gene has been generated by the *Saccharomyces* Genome Deletion Project and has turned out to be viable. We have analyzed the methyl proteins in the YPL157W mutated and wild strains by *in vivo* labeling with *S*-adenosyl-L-[*methyl*-^{3}H]methionine. The methyl protein pattern in the mutated strain appeared similar to that of wild type, but we cannot rule out the possibility that the YPL157W protein methylates a less abundant protein not identified by SDS/PAGE gel (Y.Z., unpublished work).

Methylation plays an important role in the regulation of gene expression (43, 44). Methylation of the promoter participates in the inactivation of gene transcription, which contributes to the silencing of tumor suppressor genes in cancers, whereas methylation of sites downstream of transcriptional initiation is associated with increased transcription (41, 44). In addition to DNA methylation, the importance of histone methylation in the transcriptional regulation is increasingly appreciated (15, 16). Although PIMT does not methylate the histone, it could be involved in transcriptional regulation by methylating other transcriptional components. Truncated PIMT, without the methyltransferase domain, still showed its ability to enhance the transcriptional activity of PPARγ and RXRα, suggesting this enzyme activity may not be crucial to transcription under transient transfection conditions. Given the oversimplified transient transfection system, the role of PIMT's methyltransferase activity in transcriptional regulation remains to be appreciated in the context of *in vivo* physiological condition.

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Identification of Protein Arginine Methyltransferase 2 As a Coactivator for Estrogen Receptor α*

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AQ: A

In an attempt to isolate cofactors capable of influencing estrogen receptor α (ERα) transcriptional activity, we used yeast two-hybrid screening and identified protein arginine methyltransferase 2 (PRMT2) as a new ERα-binding protein. PRMT2 interacted directly with three ERα regions including AF-1, DNA binding domain, and hormone binding domain in a ligand-independent fashion. The ERα-interacting region on PRMT2 has been mapped to a region encompassing amino acids 133–275. PRMT2 also binds to ERβ, PR, TRβ, RARα, PPARγ, and RXRα in a ligand-independent manner. PRMT2 enhanced both ERα, AF-1, and AF-2 transcriptional activity, and the potential methyltransferase activity of PRMT2 appeared pivotal for its coactivator function. In addition, PRMT2 enhanced PR-, PPARγ-, and RARα-mediated transactivation. Although PRMT2 was found to interact with two other coactivators, the steroid receptor coactivator-1 (SRC-1) and the peroxisome proliferator-activated receptor-interacting protein (PRIP), no synergistic enhancement of ERα transcriptional activity was observed when PRMT2 was coexpressed with either PRIP or SRC-1. In this respect PRMT2 differs from coactivators PRMT1 and CARM1 (coactivator-associated arginine methyltransferase). These results suggest that PRMT2 is a novel ERα coactivator.

AQ: B

Fn1 The estrogen receptor (ER)[1] is a transcription factor that belongs to the nuclear receptor superfamily (1, 2). Upon estrogen binding, ER regulates the transcription of specific target genes by binding to specific DNA response elements referred to as estrogen response elements (EREs) in their promoters or by interacting with other transcription factors such as Jun and Fos (1, 2). In addition to hormone-mediated activation, ER is also activated by growth factors including epidermal growth factor and insulin-like growth factor-1 probably through phosphorylation (3, 4). ER contains two transcriptional activation function (AF) domains: AF-1 located in the N terminus and the ligand-dependent AF-2 located in the ligand binding domain (5). The ability of AF-1 and AF-2 to activate transcription varies according to the promoter context and the cell type (6). There are two isoforms of estrogen receptors, namely ERα and ERβ (7). ERα and ERβ recognize identical DNA elements and have similar affinity for a certain estrogen, but exhibit distinct tissue distribution (7). Evidence provided by gene knock experiments indicates that ERα is the receptor responsible for the estrogen-induced growth of mammary gland and of the reproductive tract (8).

AQ: C

The precise mechanism by which ER modulates cell- and gene-specific transcription is not fully understood. Recent evidence suggests that ER activates transcription by recruiting coactivators that appear to act by modifying chromatin structure or facilitating the formation of transcriptional initiation complexes (9, 10). Among a growing list of cofactors that regulate nuclear receptors, including ER, are the well studied coactivators of the SRC-1 family (9), CREB-binding protein (CBP/p300) (11, 12), and PBP (13). PBP is a component of the thyroid hormone receptor-associated protein (TRAP)/vitamin D_3 receptor-interacting protein (DRIP) complexes (14–16). Both SRC-1 and CBP/p300 have intrinsic histone acetyltransferase activity and recruit other acetyltransferases (17–20). The acetylation of histone results in the modification of chromatin and increases the access of the DNA to other components of transcription apparatus. The multiprotein TRAP/DRIP complexes exhibit no intrinsic histone acetyltransferase activity and appear to function through the direct interaction with general transcriptional machinery (15, 16). The observation that certain coactivators such as SRC-3 (AIB1; ACTR, p/CIP, RAC3) (21–24), AIB3 (PRIP, ASC2, RAP250, NRC, TRBP) (25–29), and PBP (30) are amplified and overexpressed in some breast cancers underscores the importance of nuclear receptor coactivators in transcriptional activation and also points to their possible role in neoplastic conversion.

Post-translational modification of proteins by arginine methylation has recently been implicated in a variety of cellular processes including nuclear receptor transcriptional regulation (31). Among the five members of protein arginine methyltransferases (PRMTs) identified thus far based on protein sequences, PRMT1 is the first identified and the predominant PMRT in mammalian cells (32). PRMT1 has been shown to interact with SRC-2 (GRIP1) and enhance the nuclear receptor transactivation function (33). Coactivator-associated arginine methyltransferase 1 (CARM1)/PRMT4 was identified by its interaction with nuclear receptor coactivator SRC-2 (GRIP1) (34). PRMT1 and CARM1 are able to methylate the histones H4 and H3, respectively, suggesting their role in modulating the chromatin structure (34, 35). In addition, CARM1 also

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AQ: I

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AQ: J

[1] The abbreviations used are: ER, estrogen receptor; ERE, estrogen responsive element; AF, activation function; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; PRMT, protein arginine methyltransferase; CARM1, coactivator-associated arginine methyltransferase 1; TK, thymidine kinase; LUC, luciferase; PPAR, peroxisome proliferator-activated receptor; PRIP, PPAR-interacting protein; PPRE, peroxisome proliferator response element; GST, glutathione *S*-transferase; SRC-1, steroid receptor coactivator-1; RXR, retinoid-X receptor; TR, thyroid hormone (T_3) receptor.

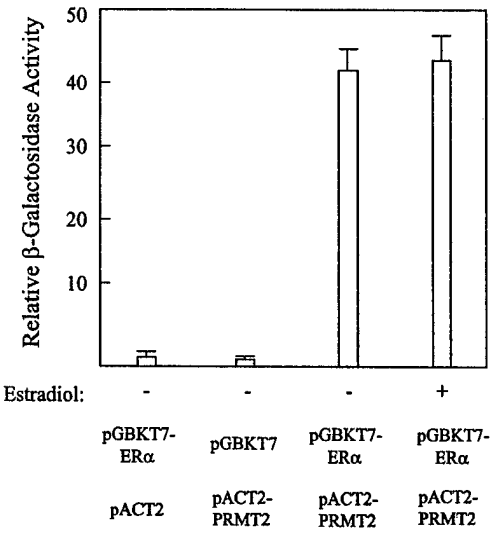

FIG. 1. **The estrogen-independent interaction of PRMT2 with ERα in yeast.** pACT2 expressing GAL4 activation domain alone, or pACT2-PRMT2 expressing the fusion protein between the GAL4 activation domain and PRMT2 was cotransformed into yeast HF7C with PGBTKT7 expressing GAL4 DNA binding domain alone or PGBKT7-ERα expressing fusion protein between GAL4 DNA binding domain and ERα into yeast HF7C. The β-galactosidase activities from equal number of cells were measured as an indication of the relative strength of interaction in the presence or absence of ligand 17β−estradiol.

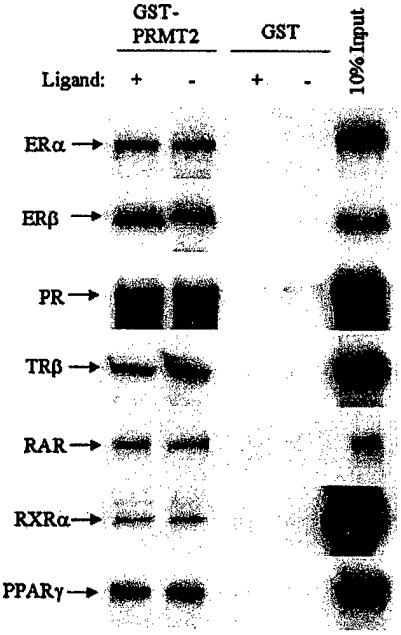

FIG. 2. ***In vitro* interaction of PRMT2 with ERα, ERβ, PR, TRβ, RARα, PPARγ, and RXRα.** GST-Sepharose beads bound with purified *E. coli* expressed GST-PRMT2 or with GST were incubated with [^{35}S]methionine-labeled ERα, ERβ, PR, TRβ, RARα, PPARγ, and RXRα in the presence (+) or absence (−) of ligand. The ligands used were: 17β-estradiol for ERα and ERβ, progesterone for PR, T_3 for TRβ, 9-*cis*-retinoic acid for RARα and RXRα, and Invitrogen 49653 for PPARγ. Following four times washing with NETN solution, the bound proteins were eluted and separated using 10% SDS-polyacrylamide gel electrophoresis and autoradiographed.

methylates the CBP/p300, which disables the interaction between CREB and CBP/p300 and blocks the CREB activation (36). PRMT2 was isolated based on its sequence similarity with PRMT1 (37). So far no methyltransferase activity has been revealed for PRMT2 (37). Here we report the identification of PRMT2 as a new ERα-binding protein through yeast two-hybrid screening. We now demonstrate that PRMT2 binds to ERα directly and also enhanced both its AF-1 and AF-2 transcriptional activity. We also demonstrate that the potential methyltransferase activity was pivotal for PRMT2 coactivator function. These results suggest that PRMT2 is a new ERα coactivator.

AQ: D

EXPERIMENTAL PROCEDURES

Plasmids—pcDNA3.1-ERα, ERE-TK-LUC, PCMX-RARα, RARE-TK-LUC, PCMV-PPARγ, and PPRE-TK-LUC and the vectors for *in vitro* translation of PR, RXRα, TRβ1 have been described elsewhere (26). ERβ is a gift from Dr. Laird D. Madison (Northwestern University). pGBKT7-ERα was constructed by inserting the full-length coding region of ERα cDNA into *NcoI*/*SalI* site of pGBKT7 (CLONTECH). PCMV-PRMT2 was an IMAGE clone purchased from Invitrogen and confirmed by sequencing. GST-PRMT2 was generated by inserting PRMT2 coding region into the *EcoRI*/*SalI* site of PGEX-5X-2. PRMT2 M, which encodes mutated PRMT2 with the mutation on the most conserved motif GCGTG (amino acids 145–149) (from GCGTG to GCRTR), was produced from PCMV-PRMT2 using a PCR site-directed mutagenesis kit (Stratagene). GST-ERα-(1−184), GST-ERα-(185−250), GST-ERα-(251−301), and GST-ERα-(302−595) were generated by inserting the corresponding ERα fragment amplified by PCR into the *EcoRI*/*SalI* site of PGEX-4T-3 and were confirmed by sequencing. Gal4-DBD-ERα-(1−184) and Gal4-DBD-ERα-(250−595) were created by inserting the corresponding fragments into *EcoRI*/*SalI* site of Gal4-DBD vector (CLONTECH) and were confirmed by sequencing. pCMV-FLAG-PRMT2, GST-PRMT1, and PRMT2ΔSH3 expression vectors encoding PRMT2 with the SH3 domain deletion are gifts from Dr. Julia Kzhyshkowska (Regensburg University).

AQ: E

Yeast Two-hybrid Screening—Yeast two-hybrid screening was performed using the matchmaker two-hybrid system kit (CLONTECH). Briefly, the yeast strain HF7C was cotransformed with a human matchmaker mammary gland cDNA expression library and pGBKT7-ERα. The positive clones were selected by their growth in medium lacking histidine and the expression of β-galactosidase in the presence of 1×10^{-7} M of 17β-estradiol.

Quantitative β-Galactosidase Assays—Appropriate plasmids were cotransformed into yeast strain HF7C, plated on selective media plates in the presence or absence of 10^{-7} M 17β-estradiol, and then incubated for 4 days at 30 °C. Ten colonies from each plate were suspended in 150 μl of buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 35 mM 2-mercaptoethanol). An equal number of cells in suspension was collected by centrifugation, and β-galactosidase activity was determined (Galacto-light kit, Tropix, Bedford, MA). Three independent assays were performed.

GST Pull-down Assays—The GST alone and GST fusion proteins were produced in *Escherichia coli* BL21 and bound to glutathione-Sepharose beads according to the manufacturer's instructions (Amersham Biosciences). *In vitro* translation was performed using rabbit reticulocyte lysate (Promega) and labeled with [^{35}S]methionine. In GST pull-down assays, a 25-μl aliquot of GST fusion protein loaded on glutathione-Sepharose beads was incubated with 5 μl of [^{35}S]methionine-labeled *in vitro* translated proteins for 2 h in 500 μl of NETN (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.7 mM EDTA, 0.05% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride). The binding was assayed in the presence or absence of specific ligands: 17β-estradiol (1×10^{-6} M) for ER; BRL49653 (1×10^{-5} M) for PPARγ; 9-*cis*-retinoic acid (1×10^{-6} M) for RXRα and RARα; Wy-14, 643 (1×10^{-5} M) for PPARα; and T_3 (1×10^{-6} M) for TRβ1, respectively. Bound proteins were washed five times with binding buffer, eluted by boiling for 2 min in 20 μl of SDS sample buffer, analyzed by SDS-PAGE, and autoradiographed.

Immunoprecipitation—COS-7 cells were transfected with 5 μg of pCDNA3.1-ERα and 5 μg of pCMV-FLAG-PRMT2 using LipofectAMINE 2000 (Invitrogen). 24 h after transfection, the cells were harvested. The lysate was immunoprecipitated with anti-FLAG (Sigma) or control serum. The precipitates were resolved by SDS-PAGE and subjected to Western blot analysis using anti-ERα.

S-adenosyl-L-methionine Binding Assay—Purified GST, GST-PRMT2, GST-PRMT2M, or GST-PRMT1 (10 μg) was incubated with 20 μCi of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (Amersham Biosciences) in the buffer (20 mM Tris-HCL, pH 7.5, 150 mM NaCl, and 2 mM $MgCl_2$) at 37 °C for 10 min. The protein was trapped on HAWP 02500 filter (Millipore). The filter was washed with the buffer (20 mM Tris-HCL, pH 7.5, 150 mM NaCl, and 2 mM $MgCl_2$) to remove unbound *S*-adenosyl-L-[*methyl*-^{3}H]methionine. The filters were dried, and the amount of bound *S*-adenosyl-L-[*methyl*-^{3}H]methionine was quantified by liquid scintilla-

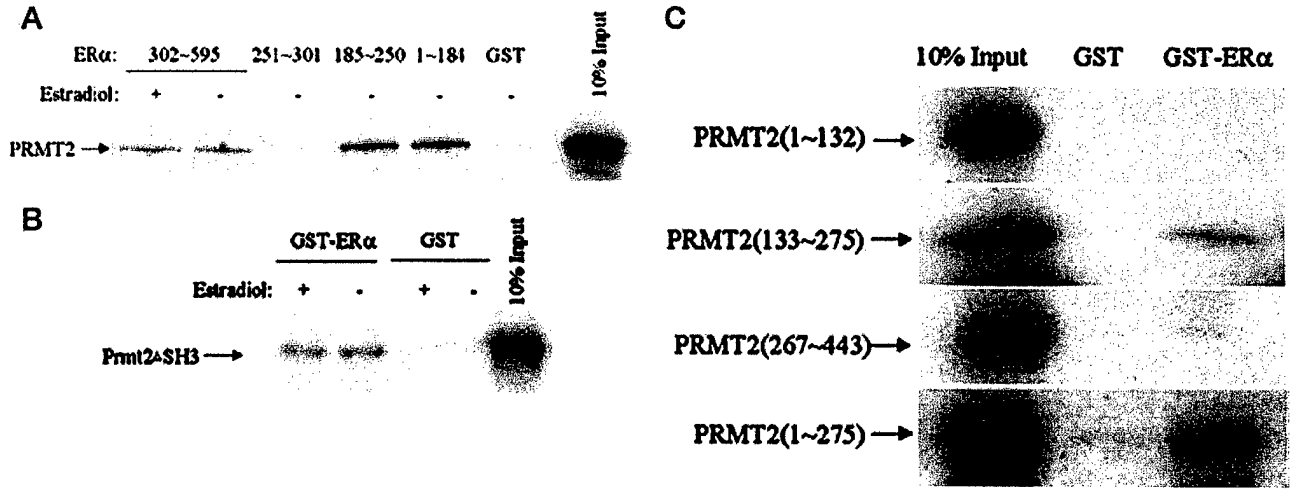

FIG. 3. **Mapping the regions for ERα and PRMT2 interaction.** *A*, GST pull-down assay was performed using [^{35}S]methionine-labeled PRMT2 and fusion proteins between GST and four different ERα fragment. Three ERα regions including AF-1 (amino acids 1–184), DNA binding domain (amino acids 185–250), and hormone binding domain (amino acids 302–595) interact with PRMT2. The binding of PRMT2 to the hormone binding domain is estrogen-independent. *B*, the SH3 domain on PRMT2 is dispensable for PRMT2 and ERα interaction. [^{35}S]methionine-labeled PRMT2 with SH3 domain deletion is able to bind to the GST-ERα fusion protein but not GST alone. *C*, the interaction region of PRMT2 to ERα was mapped to fragment from amino acid 133 to 275 by GST pull-down assay using [^{35}S]methionine-labeled truncated PRMT2 and GST-ERα fusion protein.

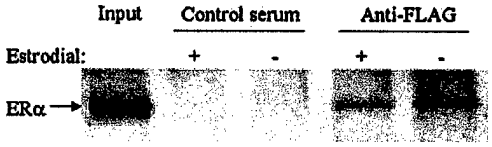

FIG. 4. **PRMT2 interacts with ERα *in vivo*.** Plasmids expressing FLAG-tagged PRMT2 and ERα were cotransfected into COS-7 cells in the presence or absence of 17β-estradiol. The cell extracts were immunoprecipitated with either anti-FLAG or control serum. The precipitates were then analyzed by Western blot using anti-ERα antibody.

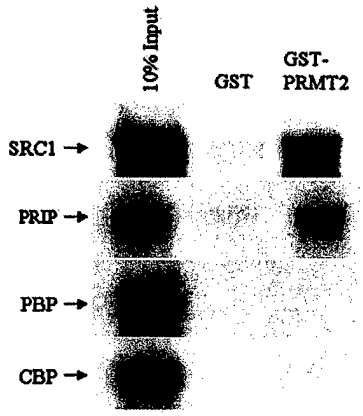

FIG. 5. **PRMT2 interacts with SRC-1 and PRIP.** GST or GST-PRMT2 fusion protein was incubated with [^{35}S]methionine-labeled SRC-1, PRIP, PBP, or CBP. Bound proteins were resolved by SDS-PAGE and detected by autoradiography.

tion counting.

Cell Culture and Transfection—CV-1 cells (1×10^5) were plated in 6-well plates and cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum for 24 h before transfection. Cells were transfected for 5 h with 1.25 μg of luciferase reporter DNA, 20 ng of plasmid expressing the receptor, and 1.25 μg of appropriate expression plasmid DNA or as indicated in the figure legends using LipofectAMINE 2000 (Invitrogen); 0.1 μg of β-galactosidase expression vector pCMVβ (CLONTECH) DNA was always included as an internal control. Cell extracts were prepared 24 h after transfection and assayed for luciferase and β-galactosidase activities (Tropix). Three independent transfections were performed for each assay.

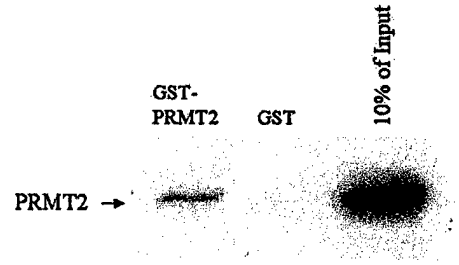

FIG. 6. **PRMT2 forms homodimer or oligodimer.** GST pull-down assay demonstrated that GST-PRMT2 fusion protein but not GST alone retained [^{35}S]methionine-labeled PRMT2.

RESULTS

Isolation of PRMT2 as an ERα-binding Protein by Two-hybrid Screening—Using full-length ERα as a bait in yeast two-hybrid system, we isolated from human mammary gland cDNA library a partial cDNA encoding PRMT2-(10–433). To examine the influence of estrogen on the interaction, pACT2-PRMT2, which was isolated by yeast two-hybrid screening and expressed as fusion protein between GAL4 activation domain and PRMT2-(10–433), or pACT2 was cotransformed with PGBKT7-ERα expressing fusion protein between GAL4 DNA binding domain and ERα or PGBTKT7 into yeast HF7C. The β-galactosidase activity was measured as an indication of the relative strength of interaction in the presence or absence of ligand. In the absence of ligand, we observed an interaction between ERα and PRMT2 that resulted in an ~40-fold increase in the β-galactosidase activity (Fig. 1). The presence of the ligand estrogen did not significantly affect the interaction between PRMT2 and ERα (Fig. 1). AQ: F F1

Interaction of PRMT2 with ERα and Other Nuclear Receptors in Vitro—The direct interaction between PRMT2 and ERα was further tested by *in vitro* GST binding assay. The immobilized GST-PRMT2, but not GST alone, retained [^{35}S]methionine-labeled ERα both in the presence and absence of estrogen (Fig. 2). Moreover, PRMT2 also showed the ligand-independent interaction with ERβ, PR, TRβ, RARα, PPARγ, and RXRα (Fig. 2). F2

To determine which region of ERα binds to PRMT2, a GST pull-down assay was performed using fusion proteins between

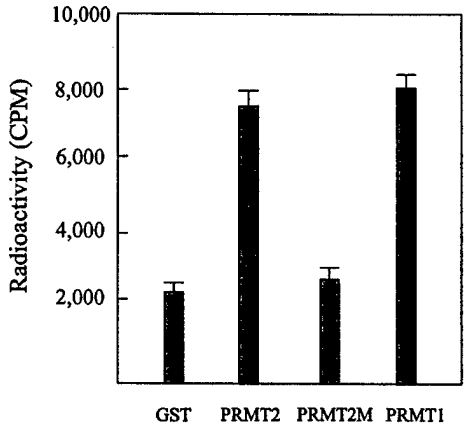

FIG. 7. **PRMT2 binds *S*-adenosylmethionine.** Purified protein was incubated with *S*-adenosyl-L-[*methyl*-^{3}H]methionine. The protein was trapped on a filter and the bound *S*-adenosyl-L-[*methyl*-^{3}H]methionine was quantified by liquid scintillation. GST-PRMT2, just as GST-PRMT1, retained a significant amount of *S*-adenosyl-L-[*methyl*-^{3}H]methionine, while the filter with GST-PRMT2M showed the same background radioactivity as that with GST alone.

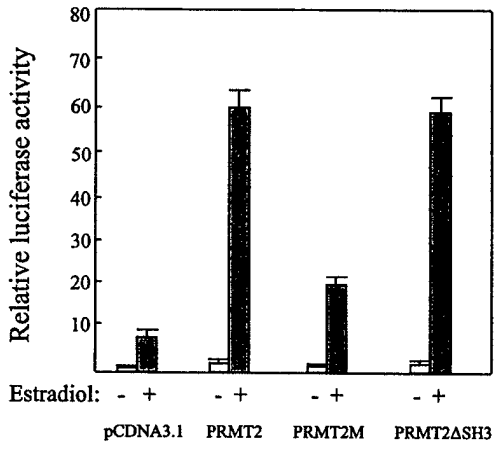

FIG. 8. **PRMT2 enhances ERα-mediated transactivation and its potential methyltransferase activity is pivotal while its SH3 domain is dispensable for this function.** 1.5 μg of PRMT2, PRMT2M, PRMT2ΔSH3 expression vector, or control vector PCDNA3.1 was transfected with 1.5 μg of the reporter construct ERE-TK-LUC and 25 ng of PCDNA3.1-ERα into CV-1 cells in the absence or presence of 10^{-7} M 17β-estradiol. The activity obtained on transfection of the ERE-TK-LUC without exogenous PPMT2 in the absence of ligand was taken as 1.

F3 GST and different regions of ERα. As shown in Fig. 3*A*, PRMT2 bound to the AF1 region, DNA binding domain, and hormone binding domain but not to the hinge region. The binding to ERα hormone binding domain was ligand-independent. The interaction between PRMT2 and ERα AF-1 region or DNA binding domain is stronger than that between PRMT2 and ERα hormone binding domain.

PRMT2 contains a Src homology 3 (SH3) domain that binds to proteins with proline-rich motif and plays a pivotal role in a wide variety of biological processes (38). A GST pull-down assay revealed that PRMT2 with an SH3 domain deletion was still able to bind to the GST-ERα fusion protein but not GST alone. Therefore, this domain is not considered necessary for PRMT2 and ERα interaction (Fig. 3*B*). The region of PRMT2 that interacts with ERα was further defined by GST pull-down assay using different truncated PRMT2 fragments. A fragment from amino acid 133 to 275 was found to interact with ERα (Fig. 3*C*).

PRMT2 Interacts with ERα in Vivo—The potential interaction between PRMT2 and ERα in the intact cell was examined by coexpressing ERα and FLAG-tagged PRMT2 in COS-7 cells followed by immunoprecipitation and Western blot analysis. As shown in Fig. 4, PRMT2 interacts with ERα both in the presence and absence of estrogen. F4

Interaction of PRMT2 with PRIP, SRC-1, and with PRMT2 itself is as follows. The potential interaction between PRMT2 and other known nuclear receptor coactivators was investigated using the GST pull-down assay. We detected the interaction of PRMT2 with PRIP and SRC-1 (Fig. 5). No interaction F5 was observed between PRMT2 and PBP or PRMT2 and CBP (Fig. 5).

The other methyltransferase PRMT1 is able to form homodimer or homooligomers (39, 40). A GST pull-down assay was performed to see if PRMT2 exhibits this property. GST and PRMT2 fusion protein but not GST alone retained [S^{35}]methionine-labeled PRMT2 suggesting that PRMT2 is capable of forming homodimer or homooligomers (Fig. 6) F6

PRMT2 Binds S-Adenosylmethionine—PRMT2 was initially isolated by its protein sequence similarity to other PRMTs and so far no methyltransferase activity has been revealed. Using bacterially expressed GST-PRMT2 fusion protein, we did not demonstrate that PRMT2 was capable of methylating histone and ERα (data not shown). We then tested the ability of PRMT2 to bind the methyl donor *S*-adenosylmethionine by a filter binding assay. Just like PRMT1, PRMT2 was found to be able to bind *S*-adenosylmethionine, whereas PRMT2 with point mutation in the *S*-adenosylmethionine binding motif (41) failed to bind *S*-adenosylmethionine (Fig. 7). F7

PRMT2 Potentiates ERα Transcriptional Activity and Its Potential Methyltransferase Activity Is Pivotal While Its SH3 Domain Is Dispensable for This Function—Having established that PRMT2 is an ERα-binding protein, we investigated the effect of increased levels of PRMT2 upon ERα transcriptional activity in CV-1 cells. The luciferase activity expressed from ERE-TK-LUC that contains one copy of ERE serves as the indicator of the ERα transcriptional activity. Expression of PRMT2 increased the estrogen-dependent transcription of luciferase gene by about 8-fold with minimal effort on basal transcription, which provided evidence that PRMT2 acts as a coactivator for ERα (Fig. 8). However, the mutated PRMT2 F8 that was incapable of binding *S*-adenosylmethionine enhanced the ERα transcriptional activity by about 2.5-fold, which is much less than the 8-fold obtained with wild-type PRMT2 indicating the importance of the potential methyltransferase activity for the role of PRMT2 as a coactivator (Fig. 8). On the other hand, PRMT2 with deletion of the SH3 domain increased ERα activity to the same extent as that for wild-type PRMT2, indicating that the SH3 domain is dispensable for its coactivator function (Fig. 8).

PRMT2 Increases Both AF-1 and AF-2 Transcriptional Activity of ERα—As ERα contains the autonomous activation domain AF-1 and ligand-dependent activation domain AF-2, we further examined the effect of increased expression of PRMT2 on their individual activities. The AF-1-(1–184) and AF-2-(251–595) were fused to GAL4 DNA binding domain, respectively, and then cotransfected with GAL4 responsive element-directed luciferase as the reporter gene. In comparison with the GAL4 DNA binding domain alone, AF-1 increased the luciferase activity by about 3-fold. The expression of PRMT2 further increased AF-1-mediated luciferase expression by about 4.5-fold (Fig. 9*A*). Therefore, PRMT2 is able to enhance F9 the ERα AF-1 activity. Just as other nuclear receptors show transcription repression in the absence of their corresponding ligands, the hormone-dependent AF-2 slightly decreased the luciferase activity without estrogen over the control. The addition of estrogen increased the AF-2-mediated luciferase expres-

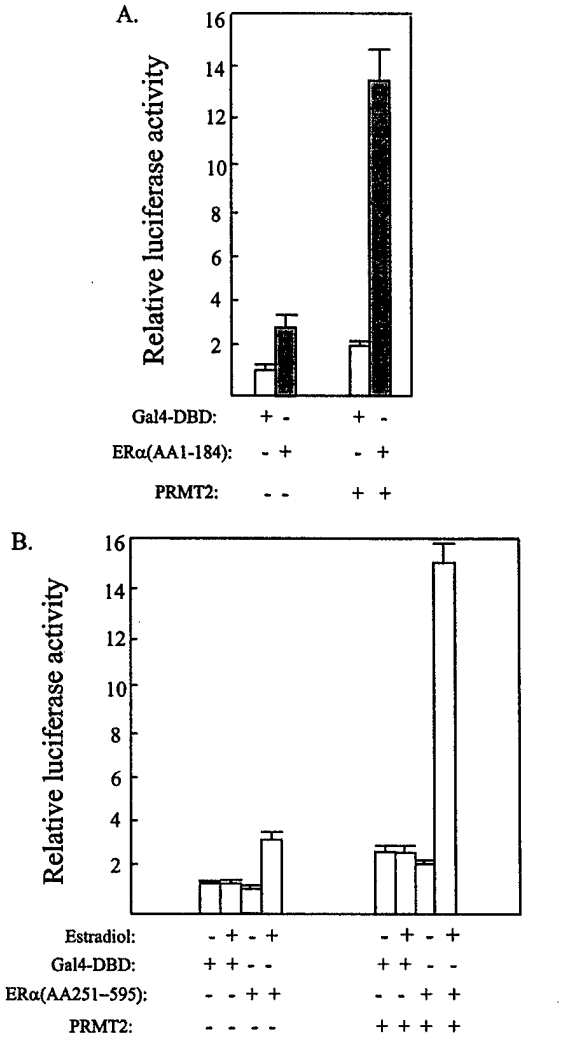

FIG. 9. **The effects of PRMT2 on AF-1 and AF-2 transcriptional activities of ERα.** Gal4-DBD-ERα-(1–184) and Gal4-DBD-ERα-(251–595) were generated by inserting the corresponding ERα cDNA fragment downstream of the Gal4 DNA binding domain (*Gal4-DBD*). The Gal4 responsive element-directed luciferase vector (Gal4-TK-LUC) was used as the reporter of activities. *A*, PRMT2 increases AF-1 activity. CV-1 cells were cotransfected with 1.5 μg of GAL-TK-LUC, 25 ng of Gal4-DBD or Gal4-DBD-ERα-(1–184), and 1.5 μg of PRMT2 expression vector or control PCDNA3.1. The activity obtained on transfection of GAL-TK-LUC and Gal4-DBD without exogenous PRMT2 was taken as 1. *B*, PRMT2 also increases AF-2 activity. CV-1 cells were cotransfected with 1.5 μg of GAL-TK-LUC, 25 ng of Gal4-DBD or Gal4-DBD-ERα-(251–595), and 1.5 μg of PRMT2 expression vector or control PCDNA3.1. The activity from transfection of GAL-TK-LUC and Gal4-DBD without exogenous PPMT2 in the absence of ligand was taken as 1.

sion by about 3-fold, which is further enhanced by coexpression of PRMT2 by about 5-fold, demonstrating that PRMT2 also potentiates the AF-2 activity (Fig. 9*B*).

No Synergistic Enhancement of ERα Activity by Coexpression of PRMT2 and SRC-1 or PRIP—Given that PRMT2 binds to SRC-1 and PRIP, we sought to determine whether there was synergistic enhancement of ERα activity by PRMT2 and SRC-1 or PRIP. In transient transfection assay with ERα and its reporter gene, PRMT2, SRC-1, and PRIP all enhanced the expression of reporter gene to different levels (Fig. 10). When PRMT2 was cotransfected with either SRC-1 or PRIP, the expression of the reporter gene luciferase was modestly decreased in comparison with PRMT2 alone (Fig. 10). Therefore, there appears no synergistic activation when PRMT2 are coexpressed with either PRIP or SRC-1.

F10

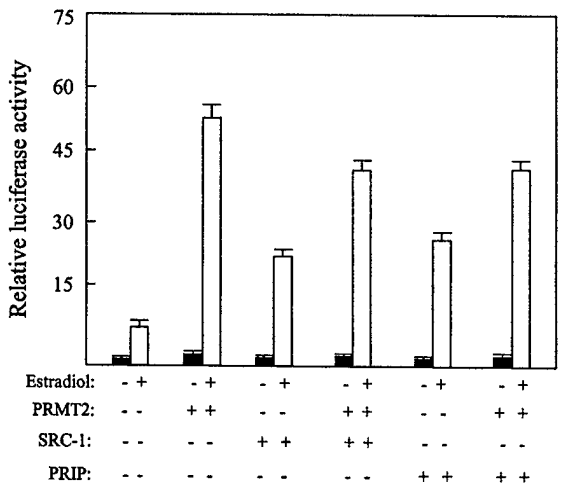

FIG. 10. **No synergistic activation was observed for coexpression of PRMT2 and SRC-1 or PRIP.** CV-1 cells were transfected with 1.5 μg of reporter construct ERE-TK-LUC, 35 ng of PCDNA3.1-ERα, and 0.8 μg of expression vector as indicated in the absence (−) or presence (+) of 10^{-7} M 17β-estradiol. Transfection without indicated expression vector was compensated by adding the same amount of PCDNA3.1. The activity obtained on transfection of ERE-TK-LUC without exogenous PPMT2 in the absence of ligand was taken as 1.

PRMT2 Also Enhances PR-, PPARγ-, and RARα-mediated Transactivation—To investigate whether PRMT2 acts as a coactivator for other nuclear receptors, transient transfection assays were performed with PR, PPARγ, and RARα responsive element-directedluciferasegene.PRMT2enhancedthePRligand-dependent transcriptional activity by about 8-fold. In comparison with ERα and PR, PRMT2, which also increased the ligand-dependent PPARγ and RARα transactivation by about 5- and 4.5-fold, respectively, showed less effect on PPARγ and RARα transactivation (Fig. 11).

F11

PRMT2 Contains No Intrinsic Transcriptional Activity—In an effort to define the mechanism by which PRMT2 acts as a coactivator, we tested if PRMT2 contains intrinsic transcriptional activity similar to that reported with other coactivators such as SRC-1 (42). PRMT2 was linked to the GAL4 DNA binding domain and transfected into CV-1 cells along with GAL4 responsive element-directed reporter gene luciferase. In comparison with GAL4 DNA binding domain alone, GAL4-PRMT2 fusion protein produced no additional activity whereas GAL4-SRC-1 fusion protein increased the luciferase activity by about 7-fold and served as a positive control (Fig. 12). Therefore, PRMT2 does not have intrinsic transcriptional activity.

F12

DISCUSSION

Using a yeast two-hybrid system with ERα as bait to screen a human mammary gland cDNA library, we isolated PRMT2 as a new ERα-interacting protein. The interaction between PRMT2 and ERα was confirmed by *in vitro* binding and *in vivo* immunoprecipitation assay. A transient transfection assay demonstrated that PRMT2 increased the ERα transcriptional activity. In agreement with the finding that PRMT2 bound to both ERα AF-1 domain and the hormone binding domain, PRMT2 enhanced both ERα AF-1 and AF-2-mediated transactivation. These results established that PRMT2 is a coactivator of ERα. However, unlike other coactivators such as SRC-1 family and CBP that show ligand-dependent binding to the nuclear receptors, PRMT2 binds to ERα both in the presence and absence of estrogen but enhances the ERα activity only with the estrogen. It appears that the interaction between ERα and PRMT2 is not enough for ERα transcriptional activation, which occurs only after the binding of estrogen resulting in

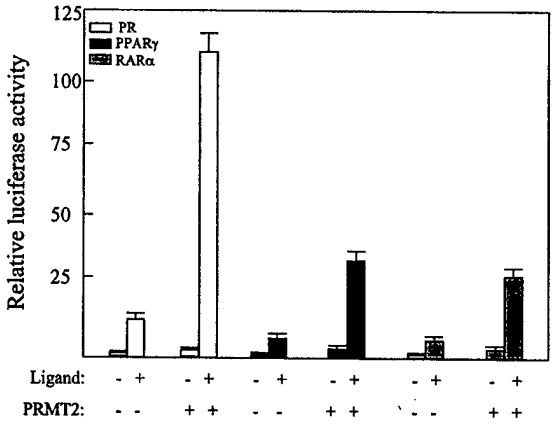

FIG. 11. **PRMT2 also enhances PR-, PPARγ-, and RARα-mediated transactivation.** CV-1 cells were transfected with 1.5 μg of reporter vector (PRE-TK-LUC for PR, PPRE-TK-LUC for PPARγ, RARE-TK-LUC for RARα), 25 ng of receptor expression vector, and 1.5 μg of control PCDNA3.1 or PRMT2 expression vector in the presence or absence of ligand (10^{-7} M progesterone for PR, 10^{-5} M BRL49653 for PPARγ, and 10^{-6} M 9-*cis*-retinoic acid for RARα). The activity obtained on transfection of the reporter vector without exogenous PPMT2 in the absence of ligand was taken as 1.

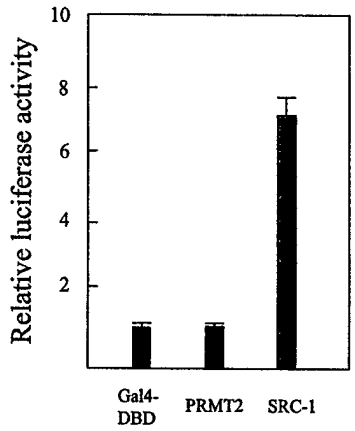

FIG. 12. **PRMT2 does not have intrinsic transcriptional activity.** 1 μg Gal4-DBD, Gal4-DBD-PRMT2, or Gal4-DBD-SRC-1 was cotranfected with 2 μg of GAL-TK-LUC into CV-1 cells. The activity of luciferase on transfection of GAL-TK-LUC with Gal4-DBD was taken as 1.

most probably the recruitment of other ligand-dependent coactivators.

Two types of PMRT activities have been identified in mammalian cells (31). Type 1 PRMT enzymes including PRMT1, PRMT3, and CARM1 catalyze the formation of monomethylarginine and asymmetric dimethylarginine. Type 2 PRMT enzymes catalyze the formation of monomethylarginine and symmetric dimethylarginine. PRMT2/JBP1 is the only type II enzyme identified so far (43). Based on the protein sequence, PRMT2 was identified as a methyltransferase most probably belonging to type I enzyme, but so far its methyltranferase activity has not been identified (37). Although we demonstrated PRMT2 is capable of binding *S*-adenosylmethionine, we failed to detect any methyltransferase activity using bacterially expressed GST-PRMT2 fusion protein with substrates including histone and ERα (data not shown). A systematic approach to identify the substrates for PRMT2 will be required, and it is also possible that some modification such as phosphorylation or some cofactor may be required for its activity. Nevertheless, the mutation in the conserved PRMT2 binding site for *S*-adenosylmethionine, which would abolish the potential methyltransferase activity, substantially diminished the PRMT2 coactivator function. The finding that PRMT2 does not have any intrinsic transcriptional activity favors the hypothesis that PRMT2 acts by modifying chromatin structure or the transcriptional apparatus through methylation. The elucidation of the substrates will be crucial for the understanding of PRMT2 coactivator function.

PRMT1 and CARM1 are two arginine methyltransferases that have been found to participate in the nuclear receptor transcriptional activation. Both PRMT1 and CARM1 interact with the carboxyl-terminal activation domain of coactivator GRIP1 and are able to methylate histones H4 and H3, respectively. PRMT1 or CARM1 enhance the nuclear receptor activity mildly by itself but substantially when coexpressed with GRIP1, suggesting that PRMT1 and CARM1 act as the secondary coactivators that are recruited by the first coactivator to modify the chromatin structure. Instead, PRMT2 strongly enhances the ERα transcriptional activity by direct interaction with ERα. Although PRMT2 was found to interact with other coactivators SRC-1 and PRIP, no synergistic activation was found with coexpression of PRMT2 and PRIP or SRC-1. The coexpression of SRC-1 or PRIP even modestly decreased PRMT2 coactivation. Therefore, although PRMT2 is a protein arginine methyltransferase highly homologous to PRMT1 and CARM1, PRMT2 may have a very different mechanism by which it acts as a coactivator, possibly because it has different substrates involved in transcriptional activation.

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